Keratin 4 Upregulation by Retinoic Acid *In Vivo*: A Sensitive Marker for Retinoid Bioactivity in Human Epidermis[1]

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Retinoids affect keratinocyte differentiation and modulate the expression of many epidermal proteins, among them cellular retinoic acid-binding protein II and the family of cytokeratins. The upregulation of the former protein is a well-known phenomenon, whereas the retinoid-induced regulation of epidermal keratin expression is more complex and only partially understood. We studied the effect of topical retinoids on the expression in healthy skin of cellular retinoic acid-binding protein II, tazarotene-induced genes 1 and 2, several epidermal keratins (K1, K2e, and K10), and two mucous keratins (K4 and K13) known to appear in epidermis under certain abnormal conditions. Reverse transcription–polymerase chain reaction experiments showed that the K4 expression was the one most overtly induced by 2 wk of open treatment with 0.05% of retinoic acid and tazarotene. Using real-time quantitative polymerase chain reaction (TaqMan) and normalization of the mRNA values to β-actin, the increase in K4 was found to be 100–1000-fold. In comparison, the expression of K13 and cellular retinoic acid-binding protein II was increased 10–50-fold, the K1 and K10 mRNA levels remained unchanged, and the K2e level decreased by a factor of 100–1000. In parallel biopsies, immunohistochemistry showed no change in K1, K2e, or K10 staining, but a strong *de novo* appearance of K4 in the granular layer after retinoid treatment. In a separate study, occlusive application of 0.025% retinoic acid in four healthy subjects produced a maximal K4 mRNA signal after 48 h and strong K4 staining after 80 h. Finally, a dose–response study showed that the *de novo* appearance of K4 can be utilized as a sensitive test for retinoid bioactivity in epidermis *in vivo*. *Key words: housekeeping genes/immunohistochemistry/quantitative real time polymerase chain reaction/tazarotene. J Invest Dermatol 114:487–493, 2000*

Vitamin A and its analogs (retinoids) unequivocally play a fundamental part both in the maintenance of normal epidermal differentiation and in the treatment of many skin diseases (see Vahlquist, 1994). In normal human epidermis the anti-keratinizing and hyperproliferative effects of topical retinoids are well known and can usually be observed histologically (Griffiths *et al*, 1993, 1996). Another, more sophisticated way of assessing the epidermal response to retinoids is to measure the expression of retinoid-regulated genes, such as cellular retinoic acid (RA) binding protein type II (CRABPII; Siegenthaler, 1990; Elder *et al*, 1993), and tazarotene-induced genes (TIG1 and TIG2; Chandraratna, 1996; Nagpal *et al*, 1996, 1997). These genes are swiftly upregulated in normal human epidermis after occlusive application of all-trans RA or its synthetic analog tazarotene. The effects of retinoids on more prominent epidermal proteins, such as cytokeratins, are more complex. Thus suprabasally expressed keratins, which are part of the normal differentiation process (e.g., K1, K2e, and K10; Fuchs, 1995), are either unaffected or downregulated by topical RA (Schweizer *et al*, 1987; Eichner *et al*, 1992; Rosenthal *et al*, 1992; Didierjean *et al*, 1996). Conversely keratins expressed in the suprabasal compartment during hyperproliferation and abnormal differentiation (e.g., K6, K13, and K16) are frequently upregulated in normal skin by topical retinoids (Eichner *et al*, 1992; Rosenthal *et al*, 1992). These effects are probably indirect, as none of the keratin genes has yet been shown to carry response elements for the retinoid receptors RARs and RXRs. Other transcription factors, however, belonging to the same superfamily of receptors (e.g., thyroid receptors and vitamin D receptor) have been shown to bind to specific DNA motifs upstream of KRT 14 and possibly also of other keratin genes (Tomic *et al*, 1990). It is conceivable that retinoids might act indirectly via heterodimerization of RAR/RXR to these and other nuclear factors (see Chambon, 1996). Furthermore, keratin synthesis may be controlled post-transcriptionally (Navarro *et al*, 1995; Bloor *et al*, 1998), making it possible for retinoids to influence the protein levels via nongenomic mechanisms also. There are at least 30 different types of epithelial keratins in the human organism, all of which fall into one of two categories: type I keratins, designated K9–K20, are acidic and are clustered on chromosome 17, whereas type II keratins, designated K1–K8, are basic and are clustered on chromosome 12 (Moll *et al*, 1984; Romano *et al*, 1988). The keratin proteins have an α-helical structure and form heterodimers with one member from each group (Fuchs and Cleveland, 1998). Whereas some keratin genes are constitutively expressed in epidermis, most of them are found in other epithelia and are only occasionally expressed in epidermis during abnormal differentiation (Fuchs, 1995). One example is K4,

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Abbreviations: CRABP, cellular retinoic acid binding protein; K, keratin; KRT, keratin gene; RA, retinoic acid; RAR, retinoic acid receptor; RXR, retinoid-X receptor; TIG, tazarotene-induced gene.

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which is normally present in esophageal and oral mucosal epithelia, but has only been noted sporadically in diseased human epidermis during RA therapy (Steijlen *et al*, 1991, 1993) and in normal human skin during long-term retinaldehyde treatment (Saurat *et al*, 1994). In an ongoing study of the mechanism of action of retinoids in bullous ichthyosis due to K1 and K10 mutations, we observed by chance a strong upregulation of K4 in both normal and lesional skin (Virtanen and Vahlquist, unpublished observation). As a search of the literature revealed no detailed information about RA-induced expression of K4 in normal human skin *in vivo*, we decided to study this phenomenon by real-time quantitative PCR (Heid *et al*, 1996) and immunohistochemistry, with the aim of establishing a useful assay for topical retinoid activity in the epidermis. A major finding was that K4 is a more sensitive indicator of retinoid activity than, for example CRABPII, TIG, and several other keratins.

MATERIALS AND METHODS

Skin specimens In the first study, three healthy volunteers (two men, one woman, age 35–51 y) were treated once daily on two sites (3 × 3 cm) of the buttocks with creams containing 0.05% all-trans RA (Aberela, Janssen-Cilag, Sollentuna, Sweden) or 0.05% tazarotene (Zorac, Allergan, Upplands Väsby, Sweden) without occlusion (about 0.35 g cream per day). Before and after 2 wk of treatment, punch and shave biopsies were taken from the test area after infiltration of the skin with lidocaine–epinephrine. The shave biopsies, typically consisting of 1–2 cm^2 of ⩾80% pure epidermis (Vahlquist *et al*, 1982), were immediately frozen and kept frozen pending RNA extraction.

In the second study, areas of gluteal skin (3 × 3 cm) were exposed to 100 μl of 0.025% RA in ethanol/propylene glycol solution (70/30 by vol.) or vehicle under occlusion for 20, 48, or 80 h, using the same procedure as in previous studies (Griffiths *et al*, 1993; Fisher *et al*, 1995). Punch and shave biopsies were taken from all of the treatment areas. In a dose–response study, gluteal skin of four healthy volunteers (three men, one woman) was exposed to RA at varying concentrations (0.00025%, 0.0025%, and 0.025% in ethanol/propylene glycol; 100 μl of each concentrations) or vehicle under occlusion for 30 h, followed by biopsy as described above. The studies were approved by the local ethical committee at Uppsala University.

RNA preparation, reverse transcription, and PCR Total RNA was extracted from shave biopsies by homogenization in TRIZOL reagent (Life Technologies, Gibco BRL, Täby, Sweden). First strand cDNA was synthesized from 3 μg of total RNA in a 30 μl reaction mixture containing oligo-$d(T)_{15}$ primer and M-MLV reverse transcriptase (Life Technologies, Stockholm, Sweden). After the reaction 30 μl RNase-free water was added to a final volume of 60 μl. The reverse transcription–PCR reaction mix included 1 × PCR buffer II, 2.5 mM $MgCl_2$, 0.2 mM of each dNTP, and 0.5 U Ampli Taq Gold polymerase (Perkin-Elmer, Stockholm, Sweden). The final concentration of primers was 0.4 μM (Life Technologies). The sequences of the studied primers are listed in **Table I**. One microliter of the reverse transcription reaction was added to a final volume of 25 μl. The reaction started with 10 min at 94°C and was then performed for 25 cycles for CRABPII and 30 cycles for K4 and TIG2, each cycle consisting of 60 s at 94°C, 75 s at 60°C, and 90 s at 72°C.

Real-time quantitative PCR (TaqMan) The PCR reaction mix contained 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 5.0 mM $MgCl_2$, 0.4 mM of each dNTP and 1.25 U AmpliTaq Gold polymerase (Perkin-Elmer). One microliter of the reverse transcription reaction was added to a final volume of 25 μl. TaqMan probes, which contained FAM as 5′-reporter and TAMRA as 3′-quencher, and oligonucleotide primers were designed with the Primer Express program (PE-Applied Biosystems, Foster City, CA). The sequences of probes and primers are listed in **Table I**. The final concentrations of the probes and primers (PE Applied Biosystems, Cheshire, U.K.) were 0.2 μM and 0.4 μM, respectively. The PCR reaction were performed for 40 cycles on an ABI Prism 7700 Sequence Detector (PE Applied Biosystems), each cycle consisting of 15 s at 94°C and 30 s at 60°C. A standard curve was generated for each template by amplifying known amounts of a PCR product at the same time as the sample. The standard curves were linear over the range of concentrations studied (data not shown). The expression of keratins and CRABPII in each sample was normalized to the expression of β-actin.

Immunostaining of keratins Skin biopsies were immediately frozen at –70°C. Six micrometer thick sections were fixed in 100% ice-cold acetone. Nonspecific binding was blocked with 10% normal horse serum (Vector Laboratories, CA). The primary antibodies were diluted 1:10 (K1, LHK1, LL017; Leigh *et al*, 1993), 1:20 (K4, 6B10, Biogenesis, Dorset, U.K.; van Muijen *et al*, 1986), and 1:1000 (K2e, IL39). The anti-serum against K10 (LH2; Perkins *et al*, 1992; Leigh *et al*, 1993) was used undiluted. The K1, K2e, and K10 antibodies were kindly donated by Professor I. Leigh, London. The antibodies were applied overnight at 4°C. Detection of the keratin antibodies by biotinylated anti-mouse IgG (1:200, Vector

Table I. PCR primers and TaqMan probes (sequences are in 5′-3′ orientation)

RT-PCR	Forward	Reverse	Product size
Keratin 4	gccatgattgccagacagc agtgt	gggggtgagcaagctctg gttg	410 bp
CRABPII	tctggcaactggaaaatcat ccga	ctctcggacgtagaccctg gt	402 bp
TIG2	agcagacaagctgccgga agag	ccctgggggctggggtctt ccact	350
Taq-Man	**Forward**	**Reverse**	**Probe**
Keratin 1	cagtggcaatctcagttccctt	gtggtggtcactgctgaactgt	atgaccctgctttgttctttcccg aga
Keratin 2e	tcttgtggaggattataagaag aagtatgag	gtaggcattgtccacgtccttt	acaaaatcattctcagcagct gtgcgctta
Keratin 4	gcagctagataccttgggcaa	cttcatacttagtcttgaagtcct ccac	ctgtcctgcatggtcttcagctc agact
Keratin 10	atgccaacatcctgcttcagat	gcagtgctacctcattctcata cttc	agctgccagcctggcattgtcg
Keratin 13	tccgtaggccttaaatctgcct	ctcctctctcctgcagggaact	ctccctctgtcttcagcacccag agga
β-actin	ctggctgctgaccgagg	gaaggtctcaaacatgatctgg gt	cctgaaccccaaggccaa ccg
CRABPII	agcagaagctcctgaagg ga	cccatcgttggtcagttctct	agggccccaagacctcgt ggac

Laboratories) was followed by use of the Vectastain ABC kit (Vector Laboratories) and 3-amino-9-ethylcarbazole (Sigma, Stockholm, Sweden).

RESULTS

The gene expressions (reverse transcription–PCR) of keratin 4, CRABPII, and TIG respond differently to retinoid treatment In a pilot study using reverse transcription–PCR, one member of the keratin family (K4) showed a biologic response suggesting that it might be a good marker for retinoid bioactivity in human epidermis. We therefore compared the induction of K4 transcription in retinoid-treated skin with that of other retinoid-responsive genes, i.e., CRABPII, TIG1, and TIG2. **Figure 1** shows the reverse transcription–PCR results in three healthy subjects before and after daily application of 0.05% of RA or tazarotene for 2 wk. Before treatment, epidermal K4 mRNA was not readily detectable in any of the samples, but after treatment with either retinoid a strong band of correct size appeared in all cases (**Fig 1*a***). In contrast, CRABPII mRNA was present before treatment and the expression was only marginally upregulated by the retinoids (**Fig 1*b***). Similarly, TIG2 mRNA was present to a variable degree in both untreated and treated samples, and only in one subject (see lanes 1–3) was there a clear-cut induction by retinoids (**Fig 1*c***). Not shown in the figure is that TIG1 mRNA was also detected in some tazarotene-treated skin samples, but RA-treated and untreated skin samples were constantly negative for this transcript (unpublished observations). Our results thus indicate that of four tested markers for retinoid activity in the epidermis, induction of K4 gene expression is a new and probably improved way of analyzing a response. We also considered the *de novo* appearance of a mucous membrane-specific keratin in retinoid-treated epidermis of biologic interest *per se* and decided to characterize further the K4 response, particularly in relation to the well-known effects of retinoids on the expression of CRABPII and other epidermal keratins.

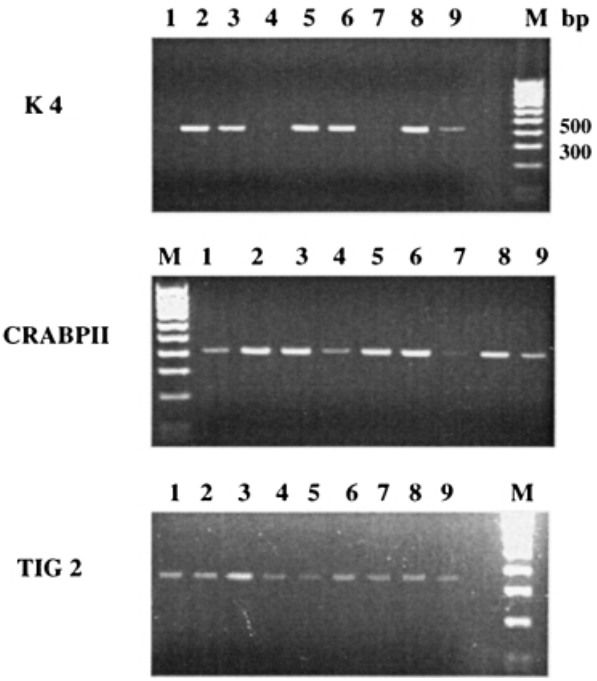

Figure 1. Induction of the mRNA expression (reverse transcription–PCR) of K4, CRABPII, and TIG2 after 2 wk of topical retinoid application in three healthy persons. *Lanes 1*, *4*, and 7 show the pretreatment results, *lanes 2*, *5*, and *8* the results after treatment with tazarotene, and *lanes 3*, *6*, and *9* the results after application of *all-trans* RA. A 100 bp ladder is shown for comparison.

Quantitative analysis of mRNA establishes the K4 gene as a unique marker for topical retinoid activity in the epidermis Using real-time quantitative PCR, the mRNA levels of CRABPII and five cytokeratins (K1, 2e, 4, 10, and 13) were analyzed in the same samples as above. Expressed as attomol per μg total RNA, the mean pretreatment levels of these transcript were 0.015, 522, 488, 0.004, 636, and 0.006, respectively. This shows that the mRNA levels of major keratins (K1, K2e, and K10) in normal epidermis exceed those of K4, K13, and CRABPII by at least four orders of magnitude. In order to investigate retinoid-induced changes in individual transcript levels we first analyzed the expression of two housekeeping genes in treated and untreated skin. As the expression of β-actin was found to be less affected by retinoid treatment than that of GAPDH (data not shown), the former gene was chosen as denominator for all other genes. As seen in **Fig 2(*a*)** tazarotene induced a 10^5-fold increase in the K4/β-actin ratio in two subjects and a 300-fold induction in a third; the response to RA was generally somewhat weaker. Similar but less apparent changes were observed in the expression of K13, the heterodimeric counterpart of K4 (**Fig 2*b***). Interestingly, the person with the highest pretherapy levels of K13 and K4 mRNA did not respond to RA and showed only a minor tazarotene-induced elevation. In **Fig 2(*c*)** the upregulation of CRABPII by retinoids is shown for comparison. The responses were interindividually quite constant, but were generally 100 times lower than the retinoid induction of

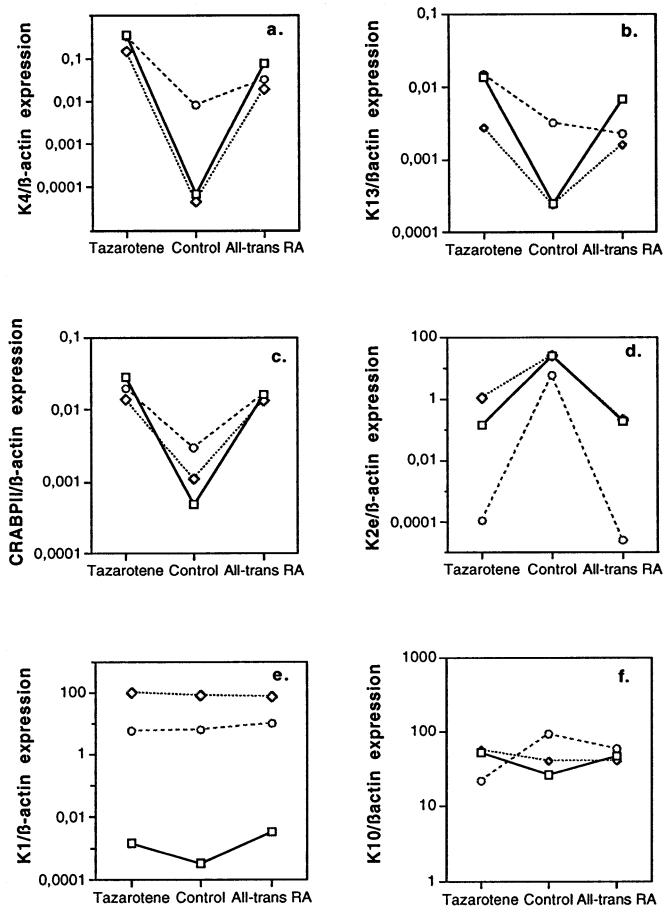

Figure 2. Retinoid-induced changes in the mRNA levels for K4, K13, CRABPII, K2e, K1, and K10 studied by real-time quantitative PCR (TaqMan). (*a*) K4, (*b*) K13, (*c*) CRABPII, (*d*) K2e, (*e*) K1, (*f*) K10. Three healthy volunteers were treated openly on two different areas with tazarotene and *all-trans* RA for 2 wk as in **Fig 1**. A sample of adjacent, untreated skin was used as control. The individual values are interconnected with lines showing the changes induced by either retinoid compared with control.

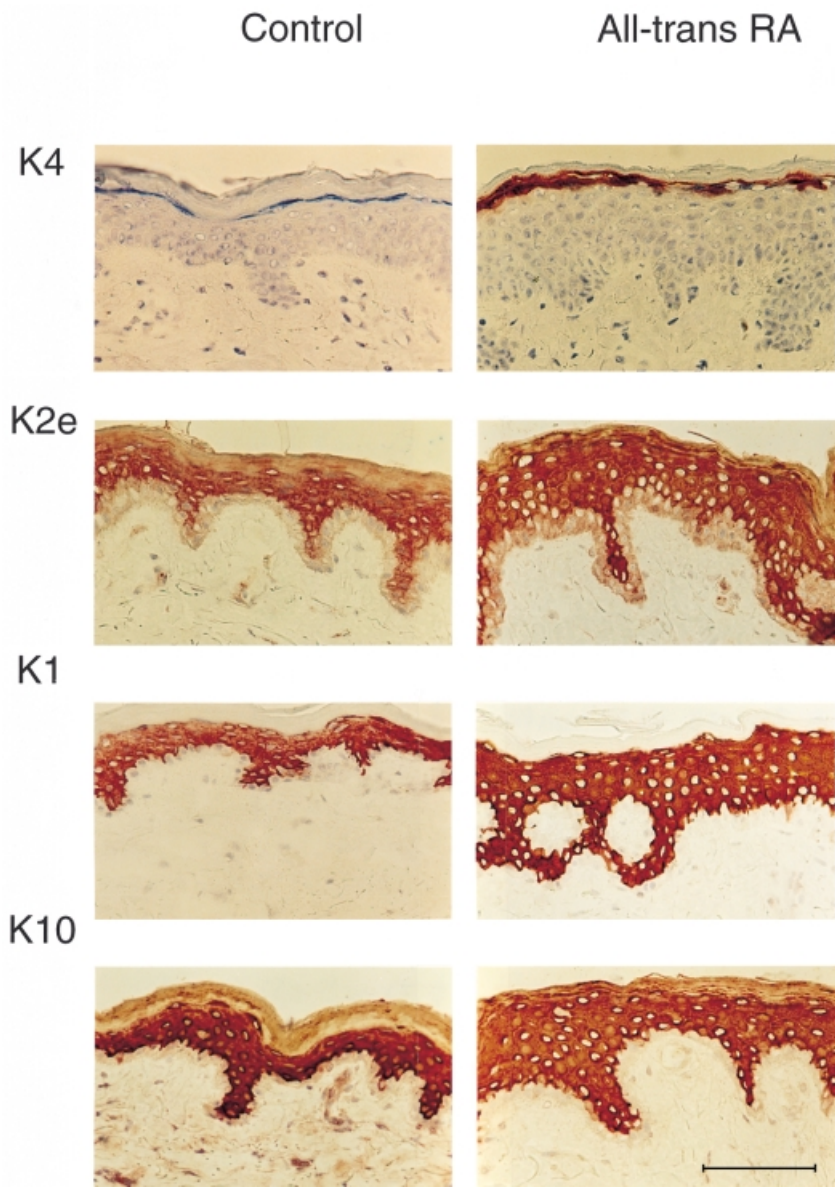

Figure 3. Immunohistochemical demonstration of epidermal keratins before and after topical treatment with RA for 2 wk. Left-hand column, before treatment; right-hand column, after treatment. Tissue sections were stained with antibodies against K1, K2e, K4, and K10 as indicated and counterstained with hematoxylin and eosin. Representative micrographs from one of three individuals treated with RA are shown. Tazarotene induced very similar results (data not shown). *Scale bar*: 50 μm.

K4 expression. As can been seen in **Fig 2** (***d–f***) the K1 and K10 expressions were not significantly affected by retinoids in our test model, but K2e was markedly downregulated by RA and tazarotene. In fact, in one man the K2e/β-actin ratio was reduced more than 100,000-fold, thus outweighing the upregulation of K4 by retinoids (see **Fig 2*a***). Downregulation of the murine epidermal keratin 70 kDa (ortholog of the human K2e) has previously been described in mouse tail treated with topical retinoids (Schweizer *et al*, 1987; Didierjean *et al*, 1996).

Immunohistochemical demonstration of altered keratin expression after retinoid treatment Highly-specific antibodies were available for K1, K2e, K4, and K10. **Figure 3** shows the immunostaining of punch biopsies obtained before and after 2 wk of topical RA treatment (same experiment as above; tazarotene results were similar). In keeping with previous results (Perkins *et al*, 1992), control epidermis was constantly negative for K4, whereas K1, K2e, and K10 antibodies stained the whole spinous layer and, in the case of K2e and K10, also the horny layer. After RA treatment an intense band of K4 staining appeared in the granular layer, but the staining patterns of K1, K2e, and K10 remained unchanged. Nevertheless, retinoid-induced epidermal hyperplasia was apparent in all treated samples. It was concluded that retinoid-induced K4 synthesis is readily demonstrable at the protein level also. We next decided to try to establish K4 induction as a short-term assay of retinoid biopotency in human epidermis simply by applying different concentrations of RA under occlusion and comparing the K4 results with those obtained with other markers.

Time-dependent and concentration-dependent induction of K4 after occlusive RA treatment: a comparison with other keratins and CRABPII Using real-time quantitative PCR and β-actin-normalized values, the time courses of the RA-induced changes in K1, K2e, K4, K13, and CRABPII expression were studied in four healthy subjects for up to 80 h after occlusion (see *Materials and Methods* for experimental details). As seen in **Fig 4** (***a***), within 20 h after application of 0.025% RA the K4 expression had increased on average 500 times and the K2e expression had reciprocally decreased almost 100-fold. In contrast, occlusion with 4% of sodium lauryl sulfate for 24 h ($n = 3$) did not affect the K4 expression but caused a variable skin irritation (data not shown). The maximal change in K4 during RA treatment was observed after 48 h and corresponded to a 10,000-fold increase in the transcript levels compared with vehicle-treated skin. In comparison, occlusive treatment with 50 μg calcipotriol per g for 96 h in five healthy individuals caused on average a $\leqslant$ 10-fold increase in K4 expression (data not shown). As also seen in **Fig 4**,

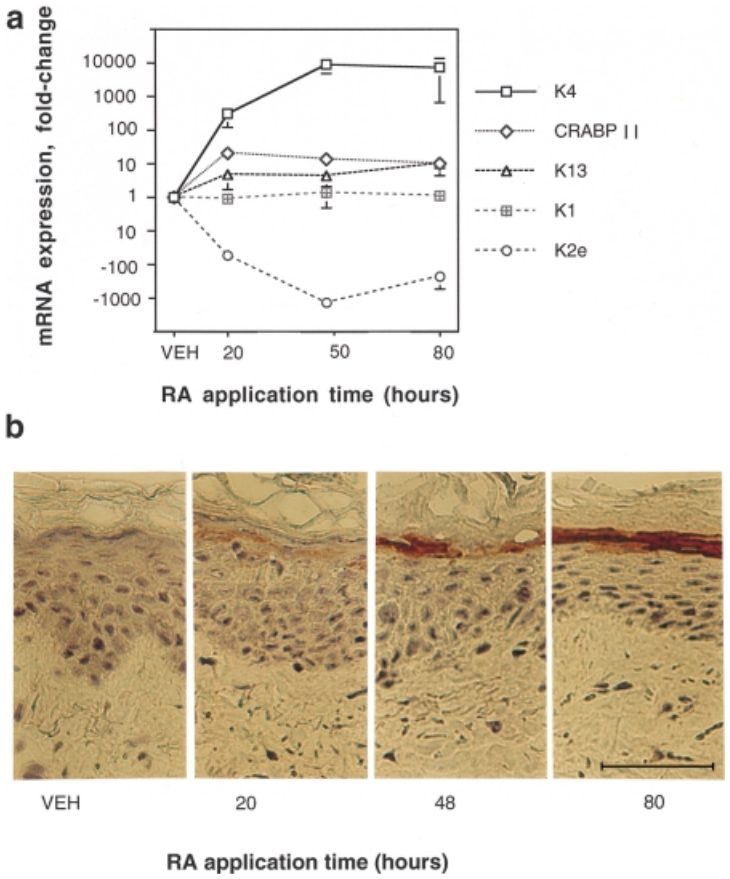

Figure 4. Time-related changes in the expression of K1, K2e, K4, K13, and CRABPII after occlusive application of 0.025% *all-trans* RA. The mRNA expression (*a*) was analyzed in shave biopsies by real-time quantitative PCR and the values were related to those in vehicle-treated skin. Data points (logarithmic scale) are mean ± SEM (n = 4). In (*b*) representative results of the immunohistochemical staining for K4 in punch biopsies taken in parallel to the shave biopsies are shown. *Scale bar*: 25 μm.

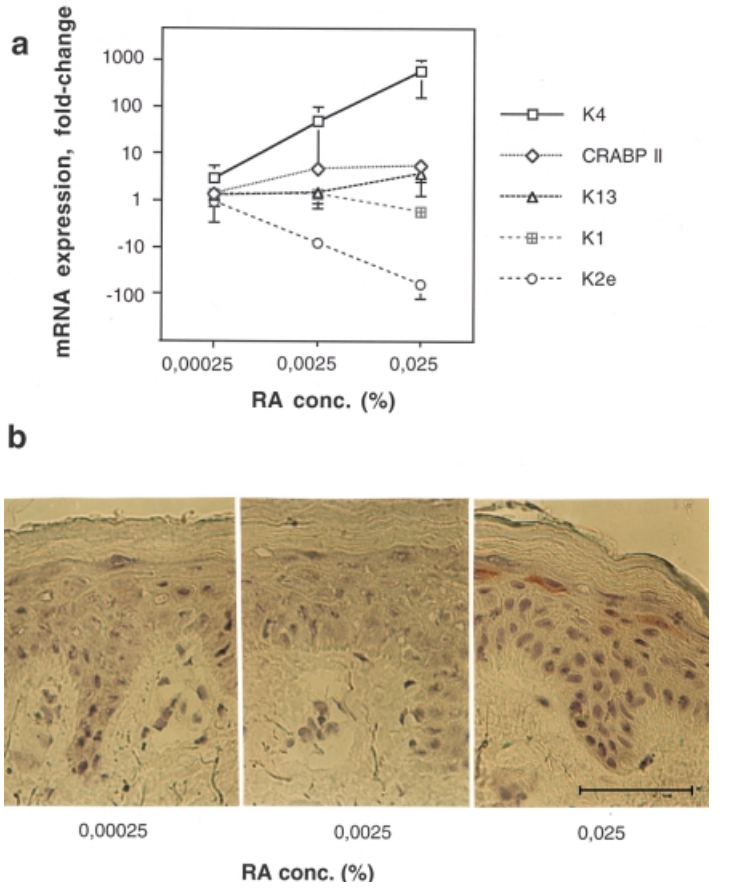

Figure 5. Concentration-dependent changes in the expression of K1, K2e, K4, K13, and CRABPII after occlusive application of varying concentrations of *all-trans* RA for 30 h. The mRNA expression (*a*) was assessed as described in **Fig 4**(***a***). Data points are mean ± SEM (n = 4). In (*b*) representative results of the immunohistochemical staining for K4 in punch biopsies are shown. *Scale bar*: 25 μm.

the expression of CRABPII and K13 increased maximally to levels 10–20 times over baseline, whereas the K1 mRNA levels remained unchanged throughout the experiment.

Parallel to the shave biopsies used for RNA extraction, punch biopsies were taken from the treatment areas and analyzed for K4 by immunohistochemistry. **Figure 4(*b*)** shows a representative time course of the induction of K4 staining by RA. Faint staining of the granular layer can be seen after 20 h, but the maximal intensity – as after 2 wk of open RA application (see **Fig 3**) – was not observed until after 80 h.

The dose–response was studied in four healthy subjects, all of whom received 30 h of occlusive treatment with RA at three different concentrations together covering a 100-fold change in concentration. All other experimental details were identical to those in the previous experiment. As shown in **Fig 5(*a*)** the increase in K4 expression was linear over the studied concentration range, with a maximum level 1000 times above baseline. Conversely, the K2e expression decreased linearly with increasing RA concentrations, eventually reaching a minimum level 100 times below baseline. The CRABPII expression increased nonlinearly up to a level 10 times higher than baseline, and the K1 and K13 expressions remained virtually constant over the RA concentration range used.

Immunohistochemistry (**Fig 5*b***) did not reveal any epidermal K4 staining at the lower RA concentrations, but at least one layer of clearly positive granular cells was seen at the highest RA concentration (same results in all four subjects).

DISCUSSION

From a biologic perspective the most pertinent finding in this study is probably the *de novo* appearance of K4 protein in normal epidermis just 20 h after a topical application of RA (**Figs 3** and **4**). Although more prolonged treatments resulted in more intense immunostaining, as in a previous study with topical retinaldehyde (Saurat *et al*, 1994), the K4 expression after RA was always restricted to the granular layer. In contrast, human buccal mucosa intrinsically expressing K4 stains most strongly in the lower layers, with progressively weaker staining toward the surface of the epithelium (Bloor *et al*, 1998). This is also true for buccal staining of K13 (Bloor *et al*, 1998), the heterodimeric counterpart of K4 in internal epithelia. Although *de novo* appearance of K13 in RA-treated epidermis has been noted previously (Rosenthal *et al*, 1992), no parallel studies of the K4/K13 mRNA and protein expressions seem to have been made before in normal human skin. Using quantitative reverse transcription–PCR we found that the retinoid-induced elevation of K4 mRNA exceeded that of K13 mRNA by several orders of magnitude (e.g., **Fig 5**). Hypothetically, an unbalanced synthesis of K4 and K13 monomers might lead to the formation of unorthodox intermediate filaments in retinoid-stimulated epidermis characterized by promiscuous liaisons between K4 and an alternative type I keratin. One such type I candidate is K10, which is already present in large amounts in the granular layer and is not markedly suppressed by retinoids (Rosenthal *et al*, 1992; this study). Further support for the promiscuity concept comes from the fact that K2e, one of the normal counterparts of K10 in non-palmoplantar human skin, is reciprocally downregulated by retinoids at least at the mRNA level (**Fig 2**), although not so evident at the protein level (**Fig 3**). K2e is a late differentiation marker, which – judging from the mRNA expression – is as abundant as K1 in normal epidermis (**Figs 2** and **3**) and probably competes with K1 during the heterodimerization process (Reichelt *et al*, 1997). It is thus conceivable that under the influence of retinoids a shortage of K2e relative to K1 and K10 might be compensated by an excessive accumulation of K4 in the granular layer. Importantly, though, it is not known for certain whether K4 is functionally integrated into the cytoskeleton of the granular cells.

From an experimental viewpoint it was rewarding to find that the K4 induction by topical retinoids was both more potent and more versatile than the CRABPII and TIG responses. For instance, monitoring of protein expression is easily done with commercial K4 antibodies, whereas no such instruments are readily available for CRABPII and TIG. Not unexpectedly, immunohistochemistry revealed that the K4 protein appeared later after retinoid application and was a less sensitive indicator of retinoid activity than the K4 mRNA expression (**Figs 4** and **5**). The K4 transcripts are readily quantifiable using real-time reverse transcription–PCR, a new and highly-specific technique (Heid *et al*, 1996). Primers and fluorescent probes adapted to this method were available for several other keratins and CRABPII, but not for the TIG. For good reproducibility of the results, all mRNA values need to be normalized to that of a housekeeping gene. On the basis of previous experiences (Wang *et al*, 1985; Törmä *et al*, 2000) and present results we chose β-actin mRNA as denominator. This is a critical decision, as housekeeping genes may also be affected by retinoid treatment (Törmä *et al*, 2000; Wu and Rees, 2000). Another critical issue is the tissue sampling. We used a method whereby the skin is cut tangentially with a razor-blade parallel to the surface (i.e., shave biopsy). In experienced hands this method produces a sheet of epidermis with only small amounts (<20%) of contaminating dermis (Vahlquist *et al*, 1982). If the mRNA expression is specifically altered in one epidermal layer, however – such as the retinoid-induced expression of K4 mRNA in the granular layer – this response may become diluted by unchanged expression in other layers of the epidermis. By virtue of the very low pretreatment levels of K4 transcripts in normal epidermis, a positive response to treatment is easier to detect than, for example, when CRABPII is used as a marker. In fact, the relative increase in K4 expression after 2 d of occlusive treatment with RA exceeded the increase in CRABPII expression by at least three orders of magnitude. Furthermore, the response was shown to be linearly related to the RA concentration in the range of 0.00025–0.025%, and was not evident after treatment with a skin irritant (sodium lauryl sulfate) or an unrelated drug (calcipotriol).

Although tazarotene seemed to be a slightly more potent K4 inducer than RA in the nonoccluded test series (**Fig 2*a***), this matter was not assessed further. Using the established method for assessing K4 mRNA induction after 30 h of occlusion, it would indeed be interesting to compare the response to many synthetic retinoids with different affinities for RARs and RXRs. It would also be interesting to find out in more detail how the K4 gene is upregulated by retinoids and why it is virtually silent in normal adult epidermis and so very active in noncornifying squamous epithelium. Could this, for instance, have to do with a difference in the intrinsic RA levels in the two types of epithelia, as previously suggested for CRABPII (Siegenthaler *et al*, 1988). Another possibility relates to differences in the tissue distribution of the nuclear transcription factor AP-2. Other investigators (Wanner *et al*, 1997) have recently shown that the K4 gene in HaCaT keratinocytes contains a functional AP-2 site. These and other functional aspects of the K4 synthesis in normal and diseased human epithelia are presently being studied in our laboratory.

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REFERENCES

Bloor B, Su L, Shirlow P, Morgan P: Gene expression of differentiation-specific keratins (4/13 and 1/10) in normal human buccal mucosa. *Lab Invest* 78:787–795, 1998

Chambon P: A decade of molecular biology of retinoic acid receptors. *FASEB J* 10:940–954, 1996

Chandraratna RAS: Tazarotene—First of a new generation of receptor-selective retinoids. *Br J Dermatol* 135 (Suppl 49):18–25, 1996

Didierjean L, Carraux P, Grand D, Sass JO, Nau H, Saurat J-H: Topical retinaldehyde increases skin content of retinoic acid and exerts biologic activity in the mouse skin. *J Invest Dermatol* 107:714–719, 1996

Eichner R, Kahn M, Capetola RJ, Gendimenico GJ, Mezick JA: Effects of topical retinoids on cytoskeletal proteins—implications for retinoid effects on epidermal differentiation. *J Invest Dermatol* 98:154–161, 1992

Elder JT, Cromie M, Griffiths CEM, Chambon P, Voorhees JJ: Stimulus-selective induction of CRABP II mRNA. A marker for retinoic acid action in human skin. *J Invest Dermatol* 100:356–359, 1993

Fisher GJ, Reddy AP, Datta SC, Kang S, Yi JY, Chambon P, Voorhees JJ: All-trans retinoic acid induces cellular retinol-binding protein in human skin in vivo. *J Invest Dermatol* 105:80–86, 1995

Fuchs E: Keratins and the skin. *Annu Rev Cell Dev Biol* 11:123–153, 1995

Fuchs E, Cleveland DW: A structural scaffolding of intermediate filaments in health and disease. *Science* 279:514–519, 1998

Griffiths C, Finkel L, Transfaglia M, Hamilton T, Voorhees J: An in vivo experimental model for effects of topical retinoic acid in human skin. *Br J Dermatol* 129:389–394, 1993

Griffiths CEM, Dabelsteen E, Voorhees JJ: Topical retinoic acid changes the epidermal cell surface glycosylation pattern towards that of a mucosal epithelium. *Br J Dermatol* 134:431–436, 1996

Heid C, Stevens J, Livak K, Williams P: Real time quantitative PCR. *Genome Res* 6:986–994, 1996

Leigh IM, Purkis PE, Whitehead P, Lane EB: Monospecific monoclonal antibodies to keratin 1 carboxy terminal (synthetic peptide) and to keratin 10 as a marker of epidermal differentiation. *Br J Dermatol* 129:110–119, 1993

Moll R, Moll I, Franke W: Differences of expression of cytokeratin polypeptides in various epithelial skin tumors. *Arch Dermatol Res* 276:349–363, 1984

van Muijen GN, Riuter DJ, Franke WW, Achtstatter T, Haasnoot WH, Ponec M, Warnaar SO: Cell type heterogeneity of cytokeratin expression in complex epithelia and carcinomas as demonstrated by monoclonal antibodies specific for cytokeratins nos. 4 and 13. *Exp Cell Res* 162:97–113, 1986

Nagpal S, Patel S, Asano AT, Johnson AT, Duvic M, Chandraratna RAS: Tazarotene-induced gene 1 (TIG1), a novel retinoic acid receptor-responsive gene in skin. *J Invest Dermatol* 106:269–274, 1996

Nagpal S, Patel S, Jacobe H, *et al*: Tazarotene-induced gene 2 (TIG 2), a novel retinoid-responsive gene in skin. *J Invest Dermatol* 109:91–95, 1997

Navarro JM, Casatorres J, Jorcano JL: Elements controlling the expression and induction of the skin hyperproliferation-associated keratin K6. *J Biol Chem* 270:21362–21367, 1995

Perkins W, Campbell I, Leigh IM, MacKie RM: Keratin expression in normal skin and epidermal neoplasms demonstrated by a panel of monoclonal antibodies. *J Cutan Pathol* 19:476–482, 1992

Reichelt J, Bauer C, Porter RM, Lane EB, Herzog V, Magin TM: Out of balance: consequences of a partial keratin 10 knockout. *J Cell Sci* 110:2175–2186, 1997

Romano V, Bosco P, Rocchi M, Costa G, Leube RE, Franke WW, Romeo G: Chromosomal assignment of human type I and type II cytokeratins genes to different chromosomes. *Cytogenet Cell Genet* 48:148–151, 1988

Rosenthal DS, Griffiths CEM, Yuspa SH, Roop DR, Voorhees JJ: Acute or chronic topical retinoic acid treatment of human skin in vivo alters the expression of epidermal transglutaminase, loricrin, involucrin, filaggrin, and keratin 6 and keratin 13 but not keratin 1, keratin 10, and keratin 14. *J Invest Dermatol* 98:343–350, 1992

Saurat JH, Didierjean L, Masgrau E, *et al*: Topical retinaldehyde on human skin: biologic effects and tolerance. *J Invest Dermatol* 103:770–774, 1994

Schweizer J, Fürstenberger G, Winter H: Selective suppression of two postnatally acquired 70 kD and 65 kD keratin proteins during continuous treatment of adult mouse tail epidermis with vitamin A. *J Invest Dermatol* 89:125–131, 1987

Siegenthaler G: Gel electrophoresis of cellular retinoic acid-binding protein, cellular retinol-binding protein and serum retinol-binding protein. *Methods Enzymol* 189:299–307, 1990

Siegenthaler G, Samson J, Bernard J-P, Fiore-Donno G, Saurat J-H: Retinoid-binding proteins in human oral mucosa. *J Oral Pathol* 17:106–112, 1988

Steijlen PM, van Happle R, Muijen GN, van de Kerkhof PC: Topical treatment with 13-cis retinoic acid improves Darier's disease and induces the expression of a unique keratin pattern. *Dermatologica* 182:178–183, 1991

Steijlen PM, Reifenschweiler DO, van Ramaekers FC, *et al*: Topical treatment of ichthyoses and Darier's disease with 13-cis-retinoic acid—A clinical and immunohistochemical study. *Arch Dermatol Res* 285:221–226, 1993

Tomic M, Jiang C-K, Epstein HS, Freedberg IM, Samuels HH, Blumenberg M: Nuclear receptors for retinoic acid and thyroid hormone regulate transcription of keratin genes. *Cell Regul* 1:965–973, 1990

Törmä H, Karlsson T, Michaëlsson G, Rollman O, Vahlquist A: Decreased mRNA levels of retinoic acid receptor α, retinoid X receptor α and thyroid receptor α in lesional psoriatic skin. *Acta Derm Venereol* 80:4–9, 2000.

Vahlquist A. Role of retinoids in normal and diseased skin. In: Blomhoff R (ed.). *Vitamin A in Health and Disease*. New York: Marcel Dekker, Inc., 1994: pp 365–424

Vahlquist A, Lee JB, Michaëlsson G, Rollman O: Vitamin A in human skin: II. Concentrations of carotene, retinol and dehydroretinol in various components of normal skin. *J Invest Dermatol* 79:94–97, 1982

Wang S-Y, LaRosa GJ, Gudas LJ: Molecular cloning of gene sequences transcriptionally regulated by retinoic acid and dibutyryl cyclic AMP in cultured mouse teratocarcinoma cells. *Dev Biol* 107:75–86, 1985

Wanner R, Zhang J, Dorbic T, Mischke D, Henz BM, Wittig B, Rosenbach T: The promoter of the HaCaT keratinocyte differentiation-related gene keratin 4 contains a functional AP-2 binding site. *Arch Dermatol Res* 289:705–708, 1997

Wu Y-Y, Rees J: Variations in epidermal housekeeping gene expression in different pathological states. *Acta Derm Venereol* 80:2–3, 2000